Chapter

Endogenous and Inhaled Nitric Oxide for the Treatment of Pulmonary Hypertension

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Abstract

Since the discovery of nitric oxide (NO) as a physiological substance produced in the endothelium, the impairment of endothelial NO production and reactivity of the pulmonary vasculature to NO have been described in animal models and patients with pulmonary hypertension (PH). The NO synthase-NO-cyclic guanosine monophosphate (cGMP) pathway is impaired in pulmonary arterial hypertension (PAH), pulmonary veno-occlusive disease (PVOD), pulmonary capillary hemangiomatosis (PCH), chronic obstructive pulmonary disease (COPD), and idiopathic pulmonary fibrosis (IPF). Pioneering clinicians conceived that NO can be administered to the lung by inhalation and used this strategy to treat PH in humans and acute hypoxic PH in animal models. Inhaled NO (iNO) selectively decreases pulmonary arterial pressure with no changes in systemic arterial pressure. When iNO diffuses into the blood, it is converted to NO_3^-, thereby losing its vasodilatory effects. NO might then be recycled in hypoxic remote organs, where NO_3^- and NO_2^- are reduced to NO. In the present chapter, the metabolic fate of iNO, based on previous air pollution research in Japan, is discussed. Then, we describe recent clinical applications of iNO in pediatric patients with various diseases, including bronchopulmonary dysplasia (BPD), persistent PH of neonates, and congenital diaphragmatic hernia (CDH). We also summarize the role of iNO in the catheterization lab, including acute vasoreactivity testing to assess prognosis, indications for specific PH therapy, and operability of congenital heart disease.

Keywords: nitric oxide inhalation, pulmonary hypertension, metabolism of nitric oxide, nitrate, pediatric, neonate

1. Introduction

In 1980, Furchgott noted that the endothelium produces and releases a vasodilatory substance named endothelium-derived relaxing factor (EDRF), which diffuses into adjacent vascular smooth muscle cells and results in vascular relaxation [1]. In 1987, EDRF was identified as nitric oxide (NO) by Ignarro [2] and Moncada [3]. Murad reported the vasodilatory effect of nitroglycerin and NO formation from nitroglycerin in 1977 [4]. However, at the time, it was not known that endogenous NO is produced and released as a physiological substance in the body, especially in the vascular endothelium.

High-temperature combustion accelerates the reaction of oxygen and nitrogen in air to generate nitrogen oxides (NOx), such as NO, NO_2, and N_2O_3. A common source of NOx is car engines, among which diesel engines have particularly high production. NO reacts with O_2 to produce NO_2, which is more toxic than NO. Thus, NOx, including NO, is considered an air pollutant. Accordingly, measuring instruments and NO gas standards with known concentrations are needed to assess NO concentrations in air. In addition, NO gas has various industrial applications, including uses in the production of chemicals, semiconductors, integrated circuits, and memory storage elements and devices. Therefore, measuring instruments for NO and the delivery of NO from gas cylinders were developed long before the discovery of NO as a physiological substance in the body. NO is now recognized as a gas and a physiological substance. Pioneering clinicians determined that "as a gas, NO can be administered to the body through the lung." It was fortunate for these clinicians who first conducted NO inhalation in humans that measuring instruments for NO and NO cylinders were available.

The present chapter discusses endogenous NO production in normal and hypertensive pulmonary vasculature, the history of NO inhalation for therapeutic use, the fate of inhaled NO (iNO), effects of iNO in remote organs other than the lung, and iNO as a therapeutic strategy in pediatrics.

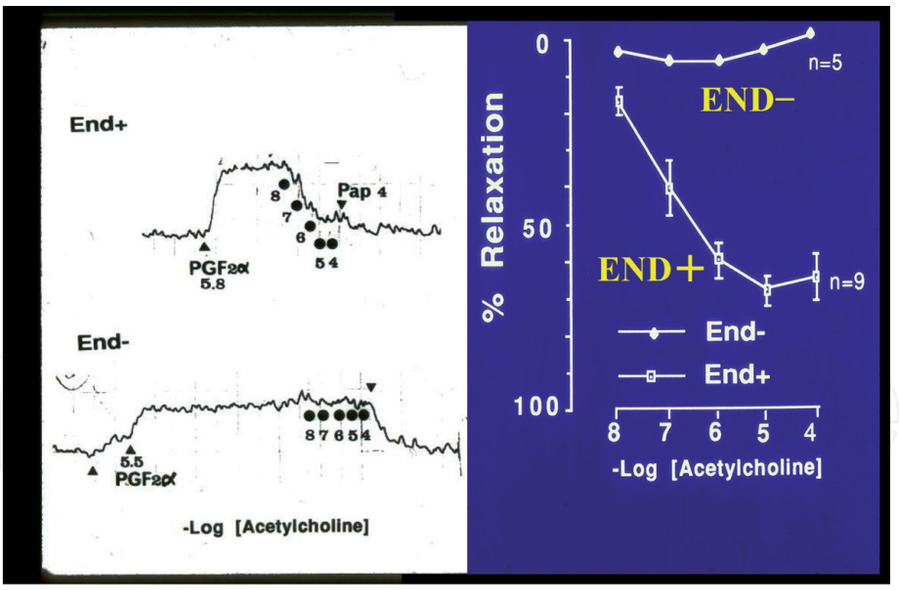
2. Endogenous NO and its role in the pathogenesis and pathophysiology of pulmonary hypertension

NO is primarily synthesized by endothelial NO synthase (eNOS, NOSIII) in pulmonary vascular endothelial cells. NO reacts with a receptor, soluble guanylate cyclase (sGC), in adjacent smooth muscle cells. Activated sGC produces cGMP, which stimulates protein kinase G (PKG) and exerts many physiological effects, including pulmonary vascular relaxation. The inhibition of NO production by L-NMMA (*N*-omega-monomethyl-L-arginine, a NOS inhibitor) decreases pulmonary flow in conscious healthy adults [5]. A deficiency in eNOS, but not iNOS or neuronal NOS, induces augmented hypoxic pulmonary vasoconstriction and a lack of endothelium-dependent vasodilation [6]. These findings support the important roles of the eNOS-NO-cyclic guanosine monophosphate (GMP) pathway in maintaining pulmonary circulation. Alteration of eNOS expression and/or function may contribute to decreased NO synthesis in pulmonary hypertension (PH). Human PH has many different etiologies. Depending on the pathological state, patients may exhibit alterations in the eNOS-NO-cGMP pathway.

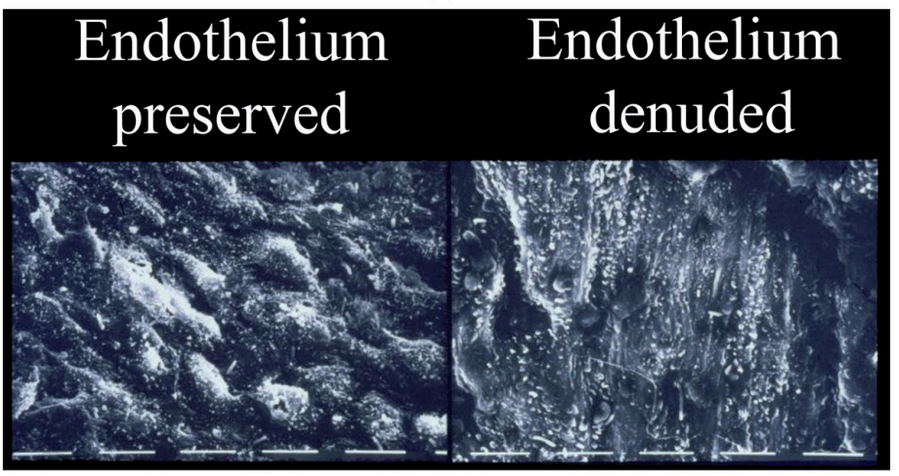
2.1 Effects of NO in isolated pulmonary arteries

The effects of NO differ among cell types. NO induces relaxation in vascular smooth muscle cells, prevents aggregation and adhesion in platelets, prevents adhesion in leucocytes, and acts as a neurotransmitter in synapses. Thus, NO regulates various cell functions. In the vasculature, NO is released from endothelial cells, reaches adjacent smooth muscle cells, and causes vascular relaxation, indicating that it functions in intercellular signaling. Among the physiological roles of endogenous NO, vascular relaxation was discovered first.

In isolated rat main pulmonary arterial rings precontracted with prostaglandin $F_{2\alpha}$ (PGF_{2a}), acetylcholine (ACh) induces relaxation in endothelium-preserved pulmonary arteries, but not in endothelium-denuded pulmonary arteries, suggesting that the endothelium in pulmonary arteries produces a relaxation-inducing substance in response to acetylcholine (**Figure 1(a)** and (**b**)).



(a)



(b)

Figure 1.
(a) Acetylcholine induces relaxation in endothelium-preserved pulmonary arterial rings. Pulmonary artery rings were obtained from normal control air rats. Rings were suspended in an 20 ml organ bath, and isometric tension was measured. Relaxation responses to acetylcholine (Ach) in endothelium-preserved (END+) and endothelium-denuded (END-) rings of the extrapulmonary artery were obtained. Endothelium was removed by gently rubbing luminal surface by fine stainless wire in endothelium-denuded rings. Rings were precontracted with prostaglandin F_{2a} (PGF_{2a}). Relaxation induced by 10^{-4} M papaverine (Pap 4) was taken as 100%. Bars mean standard error. Relaxation responses to ACh were abolished in the endothelium-denuded pulmonary arterial rings, showing that pulmonary vascular endothelium releases vasorelaxation substance named endothelium-derived relaxing factor (EDRF). The absence of the endothelium was confirmed by scanning electron micrography (b). END−, endothelium-denuded rings; END+, endothelium-preserved rings; 8, 10^{-8} mol/L, the same for 7, 6, 5, and 4. (B) Scanning electron micrograph of the endothelium-preserved pulmonary artery and endothelium-denuded pulmonary artery. Endothelium was removed by gently rubbing luminal surface by fine stainless wire. Left: luminal surface of the endothelium-preserved pulmonary artery. Right: luminal surface of the endothelium-denuded pulmonary artery.

In pulmonary arteries isolated from experimental PH models (chronic hypoxic PH in rat), the relaxation response to acetylcholine (ACh) is depressed, as observed in endothelium-denuded arteries, suggesting that endothelial function is impaired in PH arteries. Both ACh- and sodium nitroprusside (SNP, an NO donor)-induced relaxations were impaired in pulmonary arteries from rats with chronic hypoxic PH, suggesting that NO-induced relaxation is depressed in hypertensive pulmonary arteries [7, 8] (**Figure 2**). However, the magnitude of impairment seems to be higher in ACh-induced endothelium-dependent relaxation than in SNP-induced endothelium-independent relaxation [7].

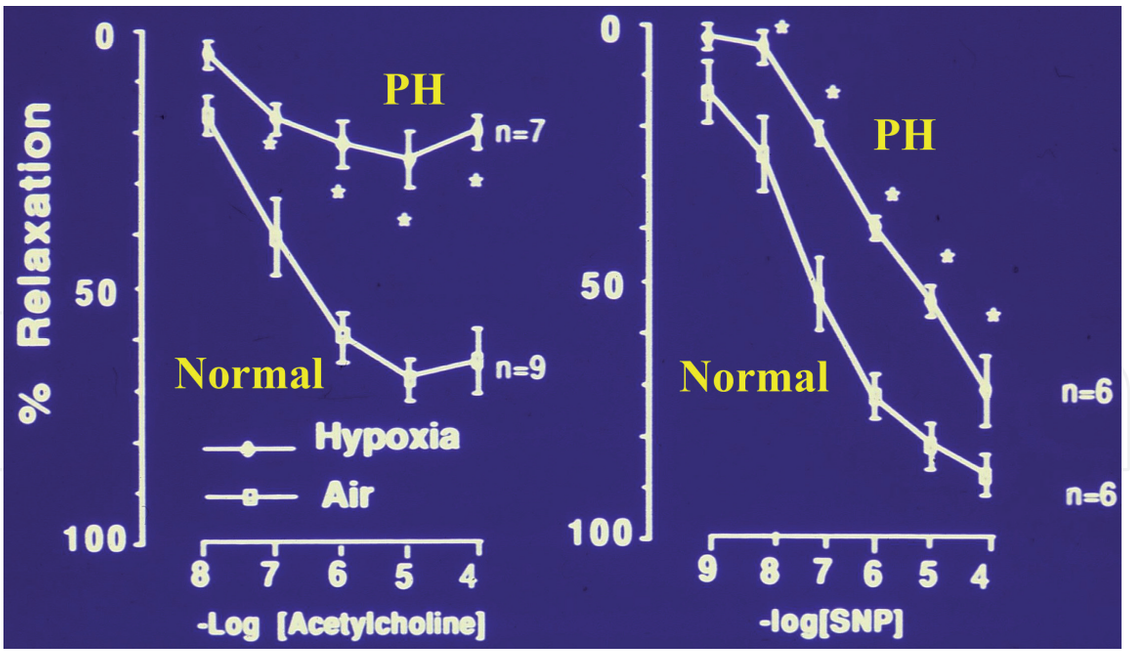


Figure 2.
The relaxation responses are depressed in isolated pulmonary arterial rings from chronic hypoxic pulmonary hypertensive rat. Pulmonary artery rings were obtained from normal control air rats and rats exposed to 10 days of hypoxia with chronic hypoxic pulmonary hypertension (PH). Isometric tension was measured. Relaxation responses to acetylcholine (ACh) in prostaglandin F_{2a} (PGF_{2a})-precontracted rings of extrapulmonary were recorded. Relaxation induced by 10^{-4} M papaverine (Pap 4) was taken as 100%. Relaxation responses to ACh were depressed in rings from rats with chronic hypoxic PH, showing that the release of vasorelaxation substance is impaired in PH rings. Although the relaxation responses to sodium nitroprusside (SNP) are impaired in pH rings compared with control, this means that there was a room where SNP could cause relaxation in PH rings from chronic hypoxic PH.

The relaxation responses to SNP are caused by the liberation of NO from SNP. To determine the vasodilatory effects of NO directly, a NO solution was made by bubbling 10% NO in pure N_2 into deoxygenated distilled water. Although depressed, the relaxation responses were indeed induced by NO in hypertensive pulmonary arteries [7] (**Figure 3**). Importantly, iNO exhibits selectivity, resulting in vasodilation in pulmonary arteries (**Figure 4**) when administered by inhalation through the trachea. The intravenous injection of NO donors simultaneously decreases both pulmonary and systemic arterial pressure.

2.2 Pulmonary arterial hypertension

2.2.1 eNOS in pulmonary arterial hypertension in humans

Although the role of the eNOS-derived NO-related pathway in pulmonary arterial hypertension (PAH) has been determined, its pathophysiology remains unclear. eNOS plays a key role in this pathway. Giaid et al. detected decreased eNOS protein expression in human lungs with PAH [9]. Additionally, exhaled NO has been found to be lower in patients with PAH than in controls [10]. Subsequent studies have reported increased eNOS protein expression in plexiform lesions in PAH [11] and increased eNOS activity in idiopathic PAH (IPAH) lungs, despite no change in NOS expression [12]. The membrane protein caveolin-1 (CAV1) is a crucial negative regulator of eNOS activity. The CAV1 expression is decreased in IPAH lungs, which might lead to persistent eNOS activation, the accumulation of dysfunctional (i.e., uncoupled) eNOS, the formation of peroxynitrite, and the impairment of PKG

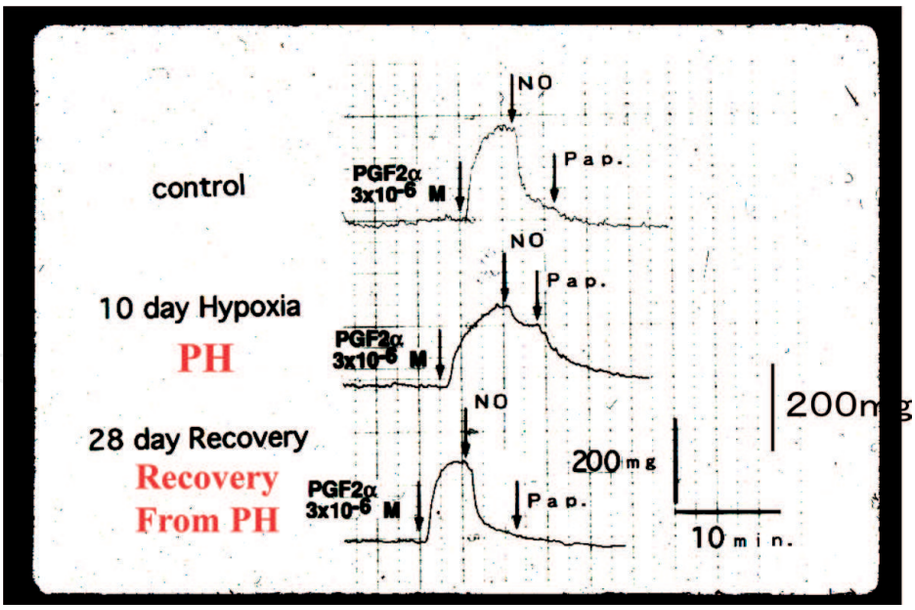


Figure 3.
NO solution (0.16–0.2 mM NO) caused the relaxation responses in isolated normal and pulmonary hypertensive arterial rings. Pulmonary artery rings were obtained from normal control air rats (A), rats exposed to 10 days of hypoxia with chronic hypoxic pulmonary hypertension (PH) (B), and rats after 28 days of recovery in room air from chronic hypoxia (C). NO solution was made by bubbling 10% NO through deoxygenated distilled water, which results in 0.16–0.2 mM concentration. Aliquots (0.5 ml) of this solution were applied to the organ bath. Papaverine (Pap) was introduced to obtain maximal relaxation. Relaxation responses to NO solution to prostaglandin F2a-precontracted rings were recorded. (A) NO-induced relaxation in pulmonary artery rings from normal rats. (B) Response to NO was depressed in pulmonary artery rings from chronic hypoxic rats. (C) The relaxation response returned to normal after 28 days of recovery from chronic hypoxic pulmonary hypertension. The result of (B) showed that NO could dilate hypertensive pulmonary vascular smooth muscles, although depressed compared to normal.

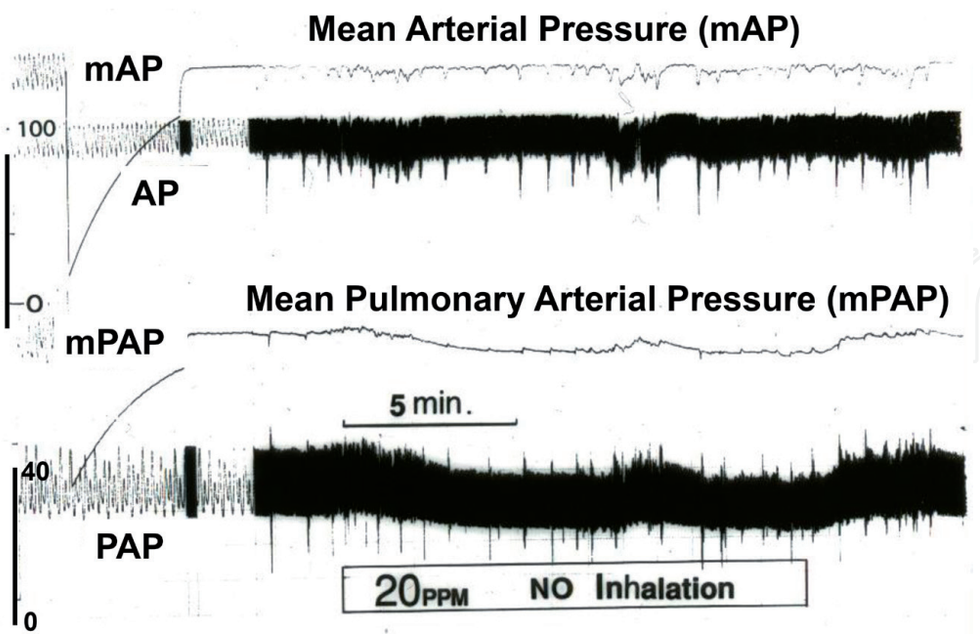


Figure 4.
Inhaled NO as selective pulmonary vasodilator in pulmonary hypertensive rats. A pulmonary artery catheter was introduced via the right external jugular vein into the pulmonary artery by a closed chest technique. Pulmonary arterial pressure(PAP) was recorded with rat fully awake in a pulmonary hypertensive rat (19 days after the single injection of monocrotaline). About 20 ppm NO inhalation decreased PAP with no change of arterial pressure. When NO inhalation was discontinued, the PAP returned to baseline.

kinase activity via tyrosine nitration [12]. Increased eNOS activity and/or expression might not be associated with NO production in PAH.

2.2.2 eNOS-NO pathway in animal models of pulmonary arterial hypertension

Animal studies using a monocrotaline (MCT)-induced PAH rat model, which is characterized by pulmonary endothelial damage and perivascular inflammation in the early pathological stage, have shown decreased eNOS expression [13] and/or phosphorylated eNOS activity [14] as well as decreases in sGC and PKG. Vasodilation induced by ACh, an endothelium-dependent NO-related vasodilator, was also impaired. Another adult rat model of severe PAH with precapillary obliterative lesion (SU/Hx model) shows similarities in the pulmonary vascular pathology to that of PAH in adults. This SU/Hx model, induced by combined SUGEN5416 (a vascular endothelial growth factor receptor II antagonist) and exposure to chronic hypoxia, showed a reduction of ACh-induced NO production and/or release in pulmonary arteries [15]. Another recent study has reported decreased CAV1 expression in the same model [16]. eNOS also translocates from cell surface caveolae to cytoplasmic and perinuclear regions in pathological state [17]. Consequently, the amount of NO production is decreased. Accordingly, the pathogenesis and progression of PAH may be partially induced by endothelial dysfunction associated with suppression of the eNOS-NO-related pathway.

2.2.3 eNOS-NO-cGMP pathway and BMPRII

Genetic variants in the eNOS-NO-cGMP pathway might cause PAH. CAV1 plays an important role in NO signaling in PAH. Mutations in CAV1 have been identified in PAH [18]. The gene encoding bone morphogenetic protein receptor 2 (BMPRII) is frequently mutated in heritable PAH [19, 20] and adult IPAH [19, 21]. BMPRII is a member of the transforming growth factor (TGF)-β receptor superfamily, localized to caveolae, and interacts with CAV1 in vascular smooth muscle cells [22, 23]. Recent studies have demonstrated that BMPRII deficiency promotes SRC-dependent caveolar trafficking defects [24]. In addition, CAV1-deficient mice have shown reduced BMPRII expression after exposure to chronic hypoxia [16]. In MCT-treated pulmonary arterial endothelial cells, BMPRII was increasingly trapped intracellularly together with increased trapping CAV1 and eNOS. These results suggest that NO-cGMP-related dysfunction and BMPRII deficiency are closely related to and play a significant role in the pathogenesis of PAH.

2.3 Pulmonary veno-occlusive disease and pulmonary capillary hemangiomatosis

Pulmonary veno-occlusive disease (PVOD), classified as a PAH subgroup, is inextricably associated with pulmonary capillary hemangiomatosis (PCH) [25]. Pulmonary vascular lesions in this condition are mainly detected in postcapillary venules and veins but are also found in pulmonary capillaries and arteries [25]. The pathogenesis is heterogeneous and poorly understood [25]. These are rare diseases, and few studies have focused on the pathogenesis and pathophysiology. Kradin et al. reported that eNOS expression in abnormal capillary lesions is significantly decreased in patients with PCH with pulmonary vascular remodeling and concomitant pulmonary hypertension and is minimally decreased or not decreased in patients without pulmonary vascular remodeling [26]. These results suggest

that the alteration of eNOS expression is associated with the pathogenesis of these complicated conditions. Further experiments are necessary to determine the precise role of the eNOS-NO-cGMP pathway in PVOD/PCH.

Biallelic mutations in eukaryotic translation initiation factor 2α kinase 4 (*EIF2AK4*) have been identified in familial and idiopathic PVOD/PCH. *EIF2AK4* encodes general control nonderepressible 2 (GCN2) [27]. The most common experimental models of these conditions are mitomycin C (MMC)-treated rats and mice [28]. Interestingly, MMC dose dependently induces pulmonary GCN2 depletion [28]. *EIF2AK4* mutations are also found in sporadic PVOD/PCH [27]. Mutation carriers have distinct histological features, including strong muscular hyperplasia of the interlobular septal vein as well as arterial severe intimal fibrosis [29]. *EIF2AK4* is activated by amino acid depletion. Because L-arginine, a substrate of NOS, is depleted during NO production, *EIF2AK4* activation can be induced by eNOS activity associated with L-arginine depletion.

2.4 Lung disease-related and/or alveolar hypoxia-induced pH

The pathophysiologic features of lung diseases include chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) and mixed pathologic diseases, including combined pulmonary fibrosis and emphysema. All involve alveolar hypoxia and subsequent hypoxic pulmonary vasoconstriction. eNOS expression is upregulated in acute hypoxia in rat lungs [30]. eNOS expression increases in a time-dependent manner in rats during the development of hypoxia-induced PH [31–33], while eNOS activity is impaired [34]. The production of tetrahydrobiopterin (BH_4), an obligatory cofactor for generating the active dimer form of eNOS, was altered in hypoxic conditions [34]. An imbalance between BH_4 and dihydrobiopterin (BH_2) may cause eNOS uncoupling and inactive monomer formation [34]. Several studies have reported decreased eNOS expression and/or activity in patients with COPD [35, 36], with severity of endothelial dysfunction correlated with degree of airflow obstruction [36]. eNOS is also absent in pulmonary arteries of patients with IPF [37]. As the histological features of this disease differ from those of COPD, the pathogenesis of IPF-induced PH may include a multifactorial and complex process involving proinflammatory cytokines and growth factors.

3. Inhaled nitric oxide

3.1 Inhaled NO as a selective pulmonary vasodilator

In 1988, at the international conference of the American Thoracic Society, Higenbottam presented his team's paper titled "Inhaled endothelium-derived relaxing factor (EDRF) in primary pulmonary hypertension (PPH)," including the first description of NO inhalation in humans with pulmonary arterial hypertension for laboratory use [38]. In 1991, Lancet published the study [39], showing that 40 ppm NO inhalation selectively reduces PAP, with no changes in systemic pressure. Frostell et al. also showed that inhaled NO (5–80 ppm) causes selective pulmonary arterial dilatation, without changes in systemic arterial pressure in sheep [40], where PAP elevation was induced by hypoxic pulmonary vasoconstriction. Both research groups referenced the studies by Yoshida, Kasama, and Kitabatake about the metabolic fate of iNO [41, 42] because toxicity and retention in the human body should be minimal. NO has been a focus in air pollution research and thus provides

a basis for work by clinicians evaluating NO inhalation in humans. In rats with chronic hypoxia- and MCT-induced PH, iNO results in a decrease in PAP with no changes in systemic arterial pressure [43–45] (**Figure 4**).

3.2 Clinical effects of iNO

iNO dilates the pulmonary vasculature by NO combining with guanylate cyclase Fe in pulmonary vascular smooth muscle cells. Most NO diffuses into the blood at alveoli, where it reacts with the Fe of oxygenated hemoglobin (oxy-Hb, O_2Hb, $O_2Hb(Fe^{2+})$) in red blood cells and is converted to NO_3^-. When NO reacts with oxy-Hb Fe^{2+} or combines with the Fe^{2+} of deoxygenated Hb (deoxy-Hb, deoxy-Hb(Fe^{2+})) in red blood cells, iNO does not have direct vasodilatory effects because it reacts or combines with Hb Fe^{2+} and becomes unable to combine with guanylate cyclase Fe of smooth muscle cells in systemic arteries. Thus, iNO is a selective pulmonary vasodilator, causing decreased PAP with no changes in systemic pressure (**Figure 4**).

The clinical use of NO inhalation is aimed at inducing selective pulmonary arterial dilation and treating PH and right ventricular failure. NO inhalation is also used to test pulmonary vascular reactivity in catheterization labs, which will be discussed in the last section of this chapter. Improved arterial oxygenation is also expected in patients with high intrapulmonary shunting [46]. Thus, the main target of iNO is lung and pulmonary circulation. In addition, iNO effects on remote organs, such as the kidney [47], liver [48, 49], heart [50], and muscle [51], have been investigated, with iNO shown to ameliorate inflammation and ischemia-reperfusion injury.

3.3 Substances that react with NO

NO reacts or combines with transition metal ions, such as thiols (–SH, –SS–, and HS–). Many enzymes and substances involved in regulating cell function include in their structure Fe, a transitional metal, thus suggesting its importance as an NO target. Because hemoglobin (Hb) and guanylate cyclase contain heme, which includes Fe in its structure, NO reacts or combines with Hb and guanylate cyclase. NO also combines with enzymes containing Fe–S in their structure, and combined NO (nitration) and Fe–S can prevent enzymatic activity. High concentrations of NO induce cell damage, which presumably result from this enzymatic dysfunction. Thus, NO is a double-edged sword. Although an appropriate amount is important for regulating cell function, an excess dysregulates cell function and causes damage.

RS–NO is a complex of SH– and NO. Nitrosothiol is a thionitrite including *S*-nitroso-albumin, where NO combines with cysteine, a component of albumin. –SH is a component of amino acids, peptides, and proteins. NO binds to –SH, forming *S*-nitrosothiol, 96% of which is *S*-nitrosoprotein. About 82% of *S*-nitrosoprotein is serum *S*-nitrosoalbumin. Thus, endogenous NO circulates in the form of *S*-nitrosoalbumin [52].

NO targets are transition metal ions, oxygen, nucleophilic centers (thiols, amides, carboxyls, and hydroxyls), and free radicals. iNO targets are (**Figure 5**) also transition metal ions, namely, in the guanylate cyclase Fe, Hb Fe, iron-sulfur (Fe-S) center. Other targets include oxygen (gaseous oxygen in the airway and alveoli), dissolved oxygen in the tissue and body fluids, the nucleophilic center of organic compounds (–S–S– and –SH), and free radicals (reactive oxygen species produced in leucocytes and macrophages). Among these substances that react with iNO, The main target of an approved medical iNO gas is guanylate cyclase Fe in pulmonary vascular smooth muscle cells.

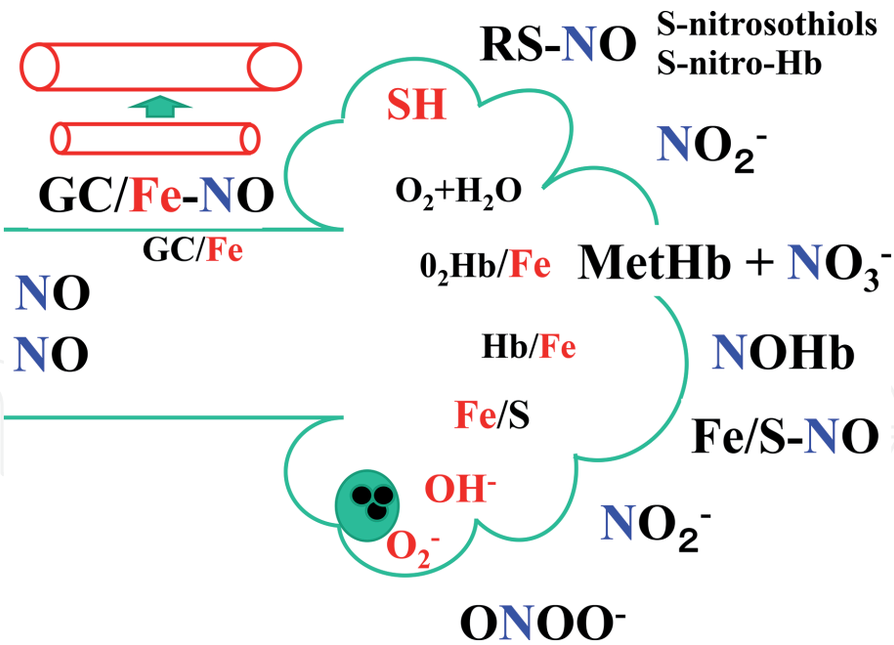


Figure 5.
The substances in the lung to react or combine with iNO. NO reacts with Fe in the guanylate cyclase (GC) and induces cyclic GMP and subsequent pulmonary vascular relaxation; NO reacts with Fe^{2+} in O_2Hb and forms MetHb and NO_3^-; NO and Fe^{2+} in deoxy-Hb combine in NOHb; NO and O_2 combine in NO_2; NO and O_2^- combine in ONOO-; NO and OH- combine in NO_2-; NO and thiol (sulfhydryl group, -SH group), amine, and iron-sulfur (Fe-S) center combine in nitrosothiol, nitrosamine, and Fe-S NO, respectively. RSH is a compound including SH group. S-nitro-Hb is combination of NO and SH in the cysteine in the Hb beta subunit. Reactive oxygen species (O_2^-, OH^-) are produced in leucocytes and macrophages.

3.4 Metabolic fate of iNO

3.4.1 Oxidation and reduction of NO

Oxidation refers to electron loss and reduction to electron gain. Reducing agents release electrons, whereas oxidizing agents receive electrons. The term redox is a combination of "reduction and oxidation reaction." Reduction and oxygenation occur simultaneously so that when a reducing agent is oxidized, an oxidizing agent is also reduced. Nitrogen monoxides involve an array of species: NO^+ (nitrosonium), NO·, and NO^- (nitroxyl anion) [53]. Among these, NO· has a single electron, and its removal forms NO^+, whereas its addition yields NO^-. NO· is electrically neutral, which contributes to its free diffusibility in aqueous medium and across cell membranes.

The main NO· targets are oxygen and transition metal ions. The various redox forms of oxygen, such as superoxide (O_2^-) and (di)oxygen, are candidates in both the gas phase and aqueous solution. Metalloproteins, such as heme-containing protein and non-heme-containing protein, and iron-sulfur clusters also react with NO·.

iNO reacts with oxy-Hb. NO oxidizes oxy-Hb to form MetHb. In other words, MetHb is oxidized oxy-Hb. Oxidized iron (MetHb) species do not catch NO, and iNO during cardiopulmonary bypass (CPB) decreases acute kidney injury [47]:

$$\underset{[\text{oxy-Hb}]}{O_2\text{Hb}(Fe^{2+})} + NO \rightarrow \underset{[\text{MetHb}]}{\text{Hb}(Fe^{3+})} + NO_3^- \qquad (1)$$

3.4.2 NO and Hb

NO reacts or combines with Hb in three ways: (1) NO combines with in the heme Fe to form NOHb (nitrosyl Hb), a metal nitrosyl species; (2) NO^- combines

with amines in Hb to form *S*-nitroso-Hb, a nitrosamine, where NO combines with cysteine in the beta subunit of Hb; and (3) O^- or NO^+ combines with the sulfhydryl center (-SH) in Hb. NO reacts with oxy-Hb and combines with deoxy-Hb. If NO reacts with oxy-Hb (Fe^{2+}), MetHb (Fe^{3+}) and nitrate (NO_3^-) are formed. If NO combines with deoxy-Hb (Fe^{2+}), NOHb (Fe^{2+}) is formed, after which NOHb (Fe^{2+}) reacts with O_2 to form MetHb (Fe^{3+}) and NO_3^-. MetHb (Fe^{3+}) is reduced to deoxy-Hb (Fe^{2+}) by MetHb reductase. The depletion of MetHb reductase or high production of MetHb causes methemoglobinemia. NO_3^- is excreted in the urine (**Figures 6**, 7).

In an in vitro experiment, Wennmalm [54] incubated NO with arterial and venous blood and measured MetHb, NOHb, NO_3^-, and NO_2^-. The reaction of NO with O_2Hb was rapid in the arterial blood (oxygen saturation 94–99%). NOHb was low in arterial blood and high in venous blood (oxygen saturation 36–86%). These results suggest that O_2Hb (oxy-Hb) gives O_2 to NO making NO_3^-. In contrast, deoxy-Hb directly combines with NO making NOHb in the absence of oxy-Hb (i.e., in the presence of deoxy-Hb). The NO and oxy-Hb reaction is completed in 100 ms [55].

3.4.3 NOHb

Nakajima and Oda have shown that the NOHb concentration is 0.13% in the blood during 20 min of 10 ppm NO inhalation [56]. This low concentration suggests the rapid turnover of NOHb, in which NOHb is presumably an intermediate in the conversion from NO to NO_2^- and NO_3^-.

3.4.4 Conversion of NO_2^- to NO_3^-

When 5 mM NO_2^- is added to human blood, NO_3^- changes are detected within 10 min [57]. The intravenous injection of sodium nitrite to rabbits results in the rapid disappearance of NO_2^-. After the intratracheal injection of $^{13}NO_2^-$, 70% of $^{13}NO_2^-$ changed to $^{13}NO_3^-$, and 26% remained as $^{13}NO_2^-$ [58]. These observations suggest that NO_2^- is converted to NO_3^- in red blood cells [59]. NO_2^- and NO_3^- are stable and unchanged in plasma without red blood cells.

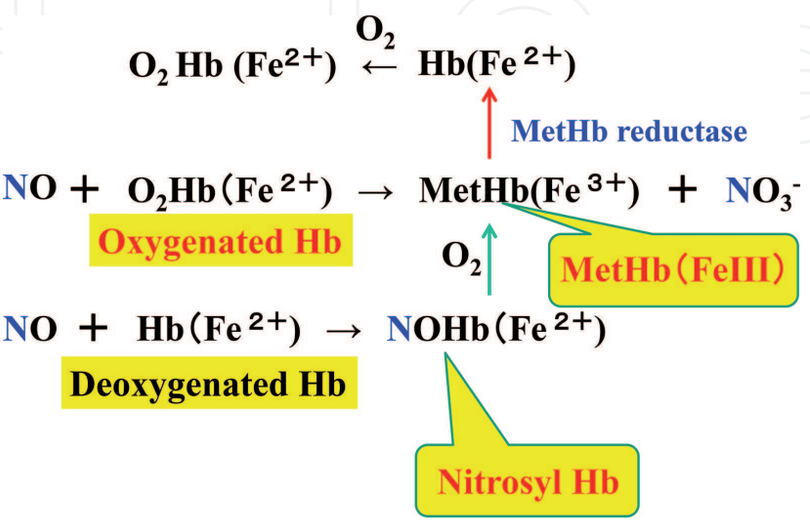


Figure 6.
Reaction of NO with Hb. NO reacts with Fe^{2+} of oxy-Hb making MetHb and NO_3^- and combines with deoxy-Hb making NOHb. NOHb reacts with O_2 making MetHb and NO_3^-.

3.4.5 No retention of iNO in the body

A clear understanding of the fate of iNO is critical for its clinical use in humans. As previously mentioned, the metabolic fate of iNO was examined in the early 1980s, and it was found that retention of iNO in the body was lacking (**Figure 7**).

An inhalation study of ^{15}NO in rats investigated the metabolism of iNO. In the carcasses, 1.6% of total inhaled ^{15}N was detected, similar to the level of natural ^{15}N. This result suggests that iNO largely does not remain in the body. About 55% of total inhaled ^{15}N was recovered in urine [41, 42], including 45% as nitrates and 10% as urea (**Figure 7**). About 10% of total inhaled ^{15}N was recovered as undetermined nitrogen compounds in feces. The remaining 35% was not recovered but is assumed to be N_2 produced from the reduction of $NO_3^- \rightarrow NO_2^- \rightarrow N_2$ by stomach flora.

3.5 iNO effects in remote organs

During cardiopulmonary bypass(CPB), hemolysis causes an increase in Hb plasma concentration due to the destruction of red blood cells. Hb includes oxy-Hb and deoxy-Hb. Oxy-Hb causes vasoconstriction, which is partly due to the depletion of NO available to induce vascular smooth muscle relaxation. NO is produced in and released from endothelial cells, some of which reaches adjacent vascular smooth muscles, causing vasorelaxation, and some of which diffuses into the plasma. If the amount of oxy-Hb in the plasma increases, the binding of NO to oxy-Hb increases, resulting in less NO reaching adjacent smooth muscle cells. Thus, the presence of large amounts of oxy-Hb might decrease NO availability in vascular smooth muscle cells (**Figure 8**).

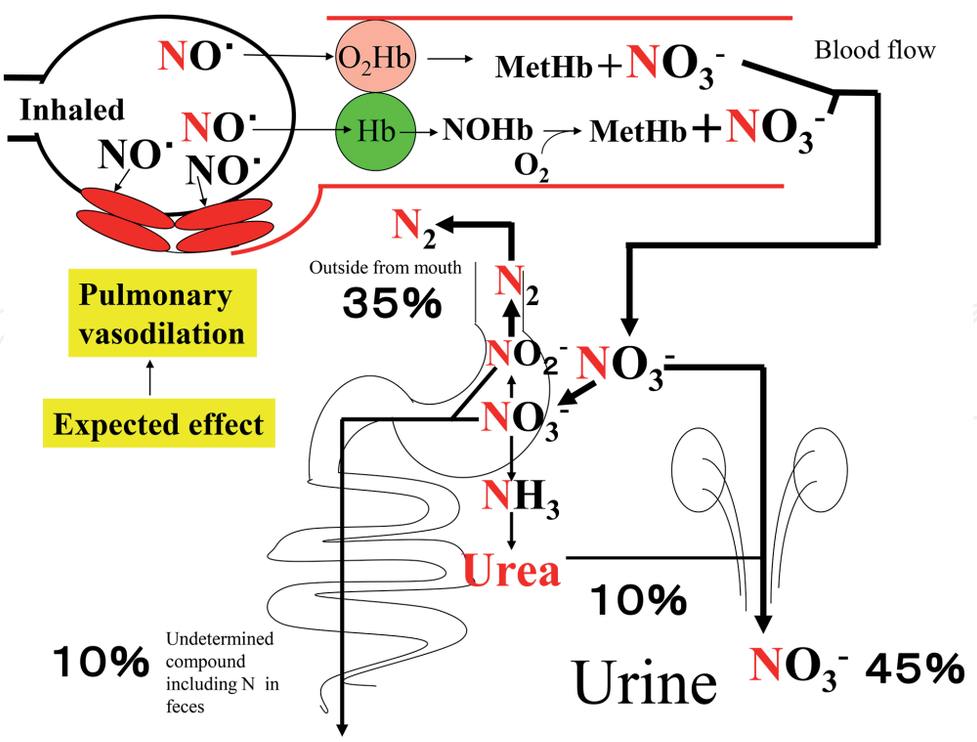


Figure 7.
Metabolic fate of NO. Almost all inhaled NO is converted to NO_3^-. Forty-five percent of NO_3^- is excreted in urine; 10% is changed to nitrogen compound except NO_3^- and NO_2^- and excreted in feces; 10% is changed to urea through the digestive tract and liver and excreted in urine. The rest will be changed to N_2 in the stomach and discharged outside of the body.

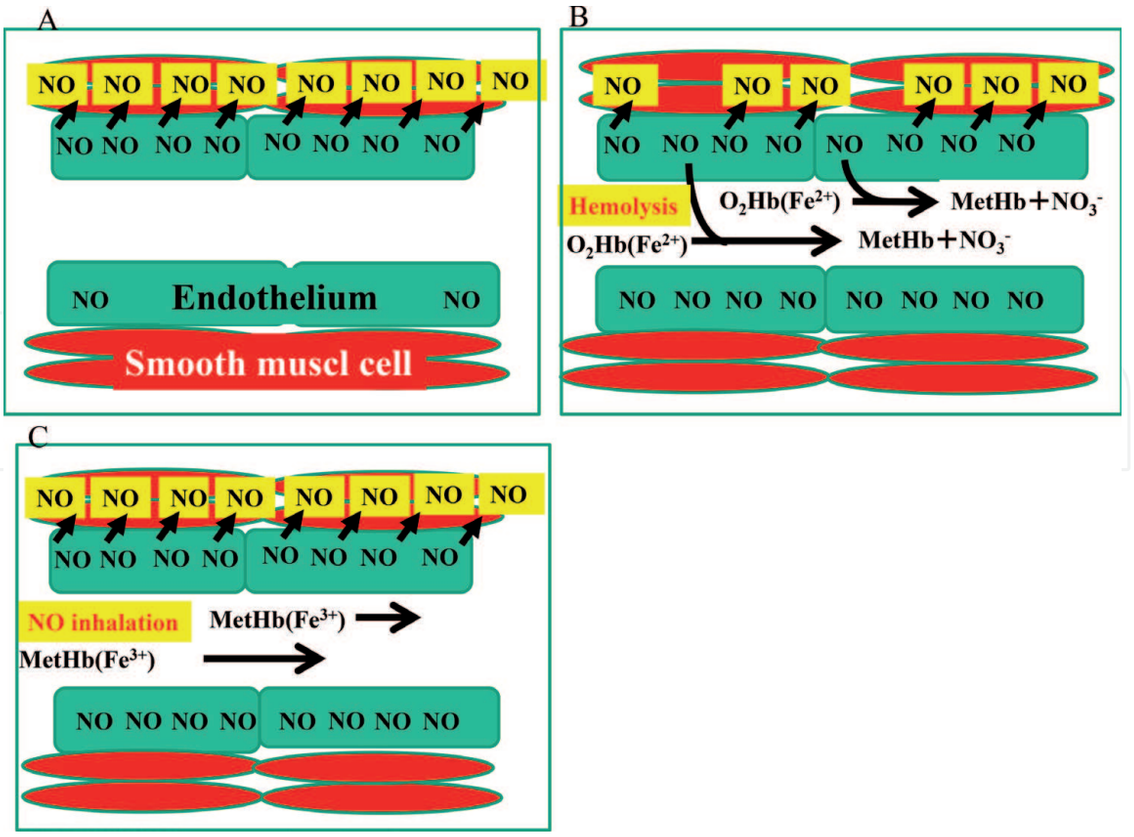


Figure 8.
NO formed in the endothelium is scavenged by plasma oxygenated Hb. (A) NO produced in the endothelium diffuses into adjacent smooth muscle cells and binds with guanylate cyclase. (B) If O_2Hb (Fe^{2+}) increases, NO produced in the endothelium diffuses into the blood and scavenged by O_2Hb (Fe^{2+}), decreasing the amount of NO diffused into smooth muscle cells. (C) If O_2 $Hb(Fe^{2+})$ is converted to $MetHb(Fe^{3+})$ by NO inhalation, NO is not scavenged, which recovers the amount of NO diffusing to the smooth muscle side.

Acute kidney injury is a common complication after cardiac surgery with prolonged CPB. Because oxygen tension is high during CPB, plasma oxy-Hb exhibits substantial hemolysis, causing vasoconstriction in the kidney. Recently, NO was demonstrated to decrease the occurrence of acute kidney injury and chronic kidney disease 1 year postoperatively [47]. NO inhalation at 80 ppm was started at the onset of CPB via a CPB machine and was continued after CPB via a mechanical ventilator for 24 h or less if patients were ready to be extubated early. Under NO inhalation, oxy-Hb was converted to MetHb, which recovered NO availability to vascular smooth muscle cells due to the decrease in oxy-Hb. Thus, exogenous NO inhalation might increase endogenous NO availability to counteract renal vasoconstriction during CPB.

In brief, the reduction of nitrite and nitrate produces NO (**Figure 9**). Nitrite is reduced by deoxy-Hb, respiratory chain enzymes, xanthine oxidoreductase, deoxygenated myoglobin, and protons, facilitating the transfer of protons to NO_2^- and thereby producing NO. These reactions are intensified under acidic and hypoxic states. After iNO is converted to NO_2^- and NO_3^-, NO can be recycled from nitrite and used to protect organs from ischemia-reperfusion injury [48, 51].

In liver transplantation, the inhalation of 80 ppm NO until reperfusion ameliorates apoptosis, attenuated increases of liver enzymes, and enhanced the recovery of coagulation factors [48].

In orthopedic knee surgery, NO inhalation prevented increases in the adhesion molecule expression on granulocytes, plasma selectin levels, and NF-κB expression in quadriceps muscles [51]. NO inhalation was started before the tourniquet application and was continued during reperfusion until the completion of surgery.

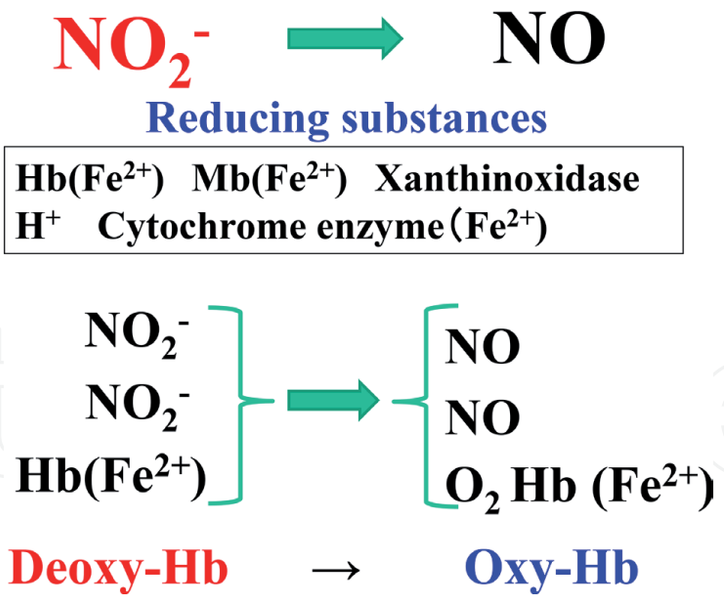


Figure 9.
Recycle of NO from nitrite and nitrate. Nitrate is reduced to nitrite, and subsequent reduction of nitrite forms NO. Reducing substances are deoxy-Hb [Hb(Fe^{2+})], myoglobin [Mb(Fe^{2+})], xanthine oxidase, hydrogen ion (H^+), and cytochrome enzymes (Fe^{2+}). NO_2^- is reduced by deoxy-Hb(Fe^{2+}) to NO showing stoichiometric relation. Please count the number of O before and after the reduction responses.

4. Update on the clinical application of iNO in pediatric patients

After NO was identified as an endothelial cell-derived relaxation factor and following preclinical studies, iNO therapy has been studied extensively in multicenter randomized trials as well as in early pilot studies of infants with severe hypoxemia associated with PH or infants with congenital diaphragmatic hernia (CDH) [60]. These studies have demonstrated improved oxygenation and reduction in the need for extracorporeal membrane oxygenation (ECMO) therapy, leading to the approval of iNO therapy by the Food and Drug Administration for use in patients at >34-week gestation with hypoxemic respiratory failure and persistent PH of the newborn (PPHN). Over the last two decades, the discussion of its application has been extended to premature infants and acute pulmonary vascular response testing to assess indications for specific pulmonary vasodilator therapy for patients with PAH or operability for children with congenital heart disease.

4.1 Role of inhaled NO in the prevention of bronchopulmonary dysplasia in premature newborns

Bronchopulmonary dysplasia (BPD), which is characterized by impaired pulmonary development resulting from insults affecting the immature lung, including inflammation, hyperoxia, and mechanical ventilation, is associated with high mortality and adverse long-term neurological and respiratory outcomes in infants born very preterm. Although the effectiveness of iNO for the treatment of PPHN is largely due to its function as a selective pulmonary vasodilator, laboratory observations also suggest other important biological effects of NO, such as roles in decreasing lung inflammation (e.g., lung vascular protein leak; pulmonary neutrophil accumulation) [61], reducing oxidant stress [62], decreasing pulmonary vascular cell proliferation [63], and enhancing alveolarization and lung growth [64–66]. These observations have led to investigations into the use of iNO to prevent the development of BPD in premature newborns. In an initial randomized,

placebo-controlled study in a single center, 7 days of iNO prevented chronic lung disease in premature infants [67]. Despite promising findings in some subsequent studies showing a reduction in BPD in premature newborns [68, 69], later trials did not confirm the beneficial effects [70]. Meta-analyses of these studies have not found evidence for a net improvement in either chronic lung disease or developmental sequelae [71], leading to the conclusion by the National Institutes of Health Consensus Development Conference [72] and the American Academy of Pediatrics Committee on the Fetus and Newborn [73] that the use of iNO to prevent BPD is not supported by available evidence [74].

4.2 iNO for the treatment of severe pulmonary hypertension in preterm infants

In addition to the use of iNO for BPD prevention, its use in preterm infants for acute management of severe hypoxemic respiratory failure has been discussed. Several case series have described responses to iNO in premature newborns with PPHN associated with prolonged oligohydramnios and pulmonary hypoplasia. Chock et al. evaluated a subset of 12 premature newborns enrolled in the Preemie Inhaled Nitric Oxide Trial with pulmonary hypoplasia after preterm premature rupture of membranes (PPROM) [75]. Six infants were treated with iNO with a mortality rate of 33% compared with 67% mortality for six infants in the placebo control group. Shah and Kluckow described outcomes for infants with PPROM and reported that survival improved from 62 to 90% after the introduction of iNO and high-frequency oscillatory ventilation [76]. Semberova et al. reported a series of 22 premature infants with a history of PPROM, pulmonary hypoplasia, and PPHN who were treated with iNO, with a survival rate of 86% [77]. Thus, iNO therapy may have important benefits in subgroups of preterm infants with severe PH, especially in patients with oligohydramnios and lung hypoplasia. Further studies of the precise effects of iNO in premature neonates are needed.

4.3 Role of iNO in newborns with congenital diaphragmatic hernia

iNO in neonates with CDH has been evaluated in three randomized trials [78–80]. Finer and Barrington performed a Cochrane Review [81] of the use of iNO for respiratory failure in infants born at or near term. They concluded that while iNO might transiently improve oxygenation, its use is not recommended for infants with CDH because the risks of a composite of either death or ECMO are similar to or worse than those of controls [82].

Based on this evidence, iNO cannot be recommended for the routine treatment of PH in patients with CDH. However, iNO continues to be regularly used for CDH. Indeed, iNO was used at some point during preoperative stabilization in 36% (191/526) of infants with CDH from the population-based CAPSNet database. The ability of iNO to improve oxygenation and reduce the need for ECMO in non-CDH patients with PH explains its continued use in patients with CDH. The lack of a response to pulmonary vasodilators in CDH is speculated to be likely due to left atrial/pulmonary vein hypertension rather than to functional changes in the pulmonary arterial vasculature [83]. A recent study suggests that the response to pulmonary vasodilators in neonates with CDH may be limited by the severity of left ventricular (LV) dysfunction and/or hypoplasia, which impairs LV filling. Careful echocardiographic assessment, therefore, may guide treatment by identifying patients who may benefit from pulmonary vasodilators, including iNO [83].

4.3.1 Acute vasoreactivity testing to assess prognosis and indications for specific PH therapy

The prognosis of children with PAH has improved in the past decade owing to new therapeutic agents and aggressive treatment strategies [84]. In idiopathic or heritable PAH (I/H-PAH), acute vasodilator testing (AVT) is recommended to identify patients who have a good long-term prognosis when treated with a long-term calcium channel blocker (CCB), accounting for 7–37% of children with PAH. For example, a >20% decrease in PAP or pulmonary vascular resistance (PVR) to inhaled NO accurately predicts a subsequent response to oral vasodilators, such as nifedipine. To identify such patients, the Sitbon criteria for positive AVT, as defined by a decrease in mean PAP by ≥10 mmHg to a value of <40 mmHg with an increased or unchanged cardiac output, is commonly used in adult I/H-PAH [85]. The Sitbon criteria can also be used to identify children who are expected to show a sustained response to CCB therapy [86]. Based on these data, the use of the Sitbon criteria is advised for AVT in children. Because only half of adult responders have a long-term hemodynamic and clinical improvement in response to CCB therapy, close long-term follow-up is required [87].

4.3.2 Acute vasoreactivity testing to assess operability of PAH associated with congenital heart disease

AVT is also used to assess operability in children with PAH associated with a systemic-to-pulmonary shunt [87, 88]. Although pulmonary vasodilators other than iNO, such as inhaled iloprost or other orally or intravenously administered compounds (e.g., sildenafil and treprostinil), can be used for AVT, iNO ± oxygen is recommended [87]. The hemodynamic change that defines a positive response to AVT in PAH

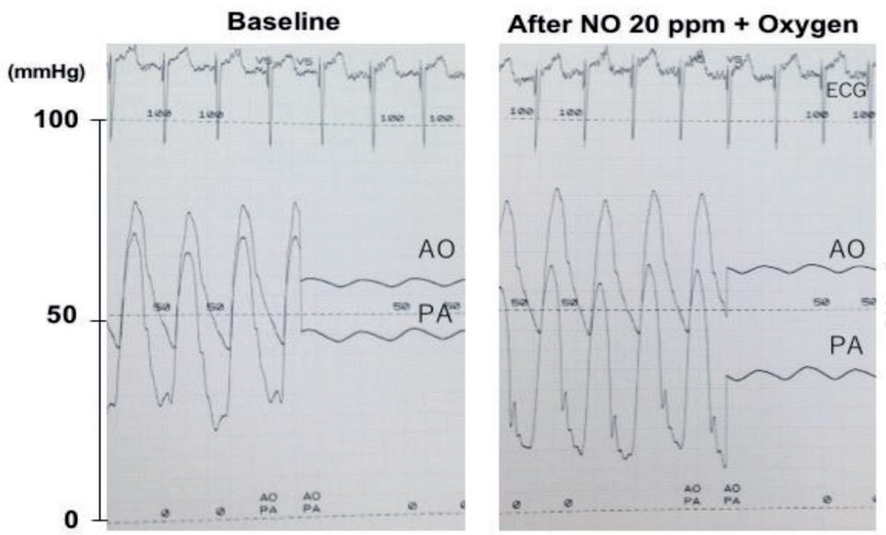


Figure 10.
*Acute vasoreactivity testingto assess operability. AVT is also used to assess operability in children with PAH associated with a systemic-to-pulmonary shunt [87, 88]. (**Figure10**, unpublished). A 5-month-old infant with Down syndrome and an atrial septal defect was evaluated for operability by acute vasoreactivity testing using inhaled nitric oxide. Pulmonary hemodynamic parameters at baseline, including pulmonary arterial pressure (76/29/49 mmHg), pulmonary vascular resistance index (6.7 Wood units m^2), the ratio of pulmonary to systemic vascular resistance (0.45), and the ratio of pulmonary to systemic blood flow (1.74), are changed to 60/14/32 mmHg, 3.14 Wood units m^2, 0.20, and 2.2 after nitric oxide inhalation, respectively. The patient underwent surgical closure of the shunt, and no postoperative pulmonary hypertension was observed. NO, nitric oxide; AO, aorta; PA, pulmonary artery.*

associated with a shunt (a ratio of pulmonary to systemic blood flow >1.5) for children should be a >20% decrease in PVR index and a ratio of pulmonary to systemic vascular resistance with respective final values of <6 Wood units m^2 and <0.3. However, specific criteria for defining a positive AVT response that predicts the reversal of PAH and good long-term prognosis have not been described. The pediatric task force of the Sixth World Symposium on Pulmonary Hypertension agreed on a general guidance for assessing operability in CHD-PAH but emphasized that the long-term impact of defect closure in the presence of PAH with increased PVR is unknown [84].

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